FUNGAL SYMBIONTS OF THE SPRUCE BARK BEETLE SYNTHESIZE THE BEETLE AGGREGATION PHEROMONE 2-METHYL-3-BUTEN-2-OL

TAO ZHAO[1,*], KAROLIN AXELSSON[1], PAAL KROKENE[2], ANNA-KARIN BORG-KARLSON[1]

[1] *Ecological Chemistry Group, Department of Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden*

[2] *Norwegian Forest and Landscape Institute, N-1431 Ås, Norway*

* Correspondence should be addressed to

E-mail: taozhao@kth.se

Tel: + 46 87908449

Fax: + 46 87912333

Abstract

Tree-killing bark beetles depend on aggregation pheromones to mass-attack their host trees and overwhelm their resistance. The beetles are always associated with phytopathogenic ophiostomatoid fungi that probably assist in breaking down tree resistance, but little is known about if or how much these fungal symbionts contribute to the beetles' aggregation behavior. In this study we determined the ability of four major fungal symbionts of the spruce bark beetle *Ips typographus* to produce beetle aggregation pheromones. The fungi were incubated on Norway spruce *Picea abies* bark, malt agar, or malt agar amended with 0.5% ^{13}C glucose. Volatiles present in the headspace of each fungus were analyzed for 7 days after incubation using a SPME autosampler coupled to a GC-MS. Two *Grosmannia* species (*G. penicillata* and *G. europhioides)* produced large amounts of 2-methyl-3-buten-2-ol (MB), the major component in the beetles' aggregation pheromone blend, when growing on spruce bark or malt agar. *Grosmannia europhioides* also incorporated ^{13}C glucose into MB, demonstrating that the fungi can synthesize MB *de novo* using glucose as a carbon source. This is the first clear evidence that fungal symbionts of bark beetles can produce components in the aggregation pheromone blend of their beetle vectors. This provides new insights into the possible ecological roles of fungal symbionts in bark beetle systems and may deepen our understanding of species interactions and coevolution in these important biological systems.

Key words: Scolytinae, bluestain fungi, plant-insect-microbe interactions

INTRODUCTION

Bark beetles are the most devastating tree-killers in conifer forests worldwide and their impact appears to be increasing with global climate change (Raffa et. al 2008). Aggregation pheromones are central to the attack strategy of tree-killing bark beetles, since pheromones coordinate the rapid mass-attacks required to overwhelm tree defenses (Blomquist et. al 2010). Another key component in the beetles' attack strategy appears to be an association with ophiostomatoid fungal symbionts that assist the beetles in exhausting tree defenses (Krokene 2015). The powerful anatomical and chemical defenses of healthy conifers deter most insect and pathogen attacks (Franceschi et. al 2005), but tree-killing bark beetles have evolved the ability to convert some of the trees' defense chemicals into beetle aggregation pheromones (Renwick et. al 1976).

Bark beetle aggregation pheromones are usually a blend of 2-3 oxygenated monoterpenes, isoprenes or other compounds (Blomquist et. al 2010). Many of these compounds are produced *de novo* in the beetles' midgut or fat body (Blomquist et. al 2010; Lanne et. al 1989), whereas some are derived from host monoterpenes, either by the beetles themselves (Renwick et. al 1976) or by symbionts in the beetle gut (Brand et. al 1975).

We still know very little about if, or how much, the beetles' fungal symbionts contribute to beetle aggregation. Knowledge about fungal volatile emission and its function in bark beetle host finding and aggregation is useful to understand conifer-bark beetle interactions and coevolution and to develop novel pest management methods against these important forest pests. In this study, we quantified pheromone production by four fungal symbionts of the spruce bark beetle *Ips typographus*, the major tree-killing bark beetle attacking Norway spruce *Picea abies*. Two symbionts (*Grosmannia penicillata* and *G. europhioides*) produced large amounts of 2-methyl-3-buten-2-ol (MB), the major pheromone component of *I. typographus*, in both bark and malt agar, indicating that fungal symbionts can *de novo* produce the beetles' aggregation pheromone..

MATERIALS AND METHODS

Fungal Symbionts

Four common fungal associates of the spruce bark beetle were used in this study (Krokene and Solheim 1996; Jankowiak 2005). *Endoconidiophora polonica* (≡ *Ceratocystis polonica*) is the most virulent of these species, with a strong ability to colonize fresh sapwood and kill trees in experimental mass-inoculations (Krokene and Solheim 1996). *Grosmannia penicillata* (≡ *Ophiostoma penicillatum*) and *G. europhioides* (≡ *O. europhioides* ≡ *O. piceaperdum*) are other primary invaders that grow well in the phloem and contribute to

phloem necrosis (Krokene and Solheim 1996; Kirisits 2004). *Ophiostoma piceae* is a less virulent secondary species that colonizes the sapwood during the later stages of beetle attack (Kirisits 2004). All fungal isolates used in this study were obtained from the culture collection of the Norwegian Forest and Landscape Institute in Ås, Norway. Isolates were maintained on malt agar (2% malt, 1.5% agar) at 4 ºC, and transferred to fresh malt agar at 25 ºC 7-10 days before the start of the experiments.

Bioassays to Detect Pheromone Production by Fungal Symbionts

The fungi's ability to produce beetle aggregation pheromones was assayed by incubating fungi on Norway spruce bark in headspace vials and analyzing emitted volatiles. We prepared bioassay units by taking 10-mm diameter bark plugs with a pre-drilled 4-mm diameter hole in the center from a spruce log, placing the bark plugs individually in 20 ml screw top glass headspace vials (Supelco, USA), and inserting a 4-mm diameter plug of sterile agar or agar colonized by each of the four fungi into the hole in the center of the plug. We prepared a total of 30 vials, with six replicates for each of the five treatments (agar with active growing mycelium of *E. polonica*, *G. penicillata*, *G. europhioides* or *O. piceae* and sterile agar control). Bark plugs were taken from a 0.5 m long log from a 48-year-old Norway spruce tree felled in early May 2013 and kept at 4 ºC at the chemical ecology laboratory at the Royal Institute of Technology, Sweden until the bioassays started two days later. When the vials had been loaded with agar/fungus plugs they were sealed by a stainless steel cap equipped with a PTFE-faced butyl septum (Supelco, USA). Volatiles present in the headspace of each vial were collected 1, 3, 5, and 7 days after incubation using an SPME autosampler. At each sampling time the autosampler inserted a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVD) SPME fiber (Supecol, PA, USA) through the septum for 50 minutes before injecting the collected volatiles into a GC-MS for separation, identification and quantification (see below).

To confirm that any MB detected in the bark plug incubation assay was *de novo* produced by fungi, we did a labelled glucose experiment with *G. europhioides,* the fungus with the highest MB production in the bark plug assay. We incubated *G. europhioides* on three different growth media: (1) malt agar (2.0% malt, 1.5% agar), (2) malt agar with 0.5% ^{13}C labeled glucose (99%, Cambridge Isotope Laboratories, Inc.) or (3) malt agar with 0.5% unlabelled glucose (99.5%, Sigma). For each type of medium six 20 ml headspace vials were filled with 3 ml medium at 50-60 °C using a sterilized plastic pipette. The vials were tilted about 30º to increase the surface area of the malt agar medium. When the malt agar had cooled to room temperature we placed a 4 mm malt agar plug colonized by *G. europhioides* at the centre of three vials with each media type. The remaining vials were used as no-fungus controls. After fungal inoculation, all vials were sealed as described above and kept at 25 °C for one week. Volatiles present in the headspace of each vial were then collected by SPME fibre as described above and analysed by GC-MS.

GC-MS Analysis

All samples were analyzed using an Agilent 7890 A GC combined with a 5975C inert MSD with triple-axis detector and a HP-5 capillary column (Agilent, 30 m, 0.25 mm id, 0.25 μm film thickness) (Agilent Technologies, CA, USA). Helium was used as the carrier gas at a constant flow of 1 ml min^{-1}, the temperature of the ion source was 150 ºC, the mass detector was operated with a mass range of 30–400, and the electron impact ionization was 70 eV. Immediately after SMPE collection, volatile samples were transferred to the injector to desorb the volatiles at 225 ºC for 5 min. MB and other volatiles in the samples were then separated using a temperature program of 40 ºC for 3 min, increasing to 160 ºC at a rate of 4 ºC min^{-1}, then to 230 ºC at a rate of 20 ºC min^{-1} and then remaining constant for 5 min. To verify the presence of MB, additional samples were collected by SPME fiber and analyzed using an Agilent 7865 GC with a different type of column (DB-wax column, Supelco; 30 m, 0.25 mm id, 0.25 μm film thickness) using the temperature program described above. MB was identified by comparing retention times and mass spectra with available authenticated standards in the HP-5 and DB-wax columns. The incoporation of ^{13}C into MB by *G. europhioides* was confirmed by comparing the mass spectra of MB from fungi growing on malt agar with 0.5% ^{13}C labelled glucose versus unlabelled glucose.

Data analysis

MB amounts emitted from the different treatments 1-7 days after incubation were subjected to repeated measures one-way ANOVA (Statistica 6.0, Statsoft Inc., USA). Data were log(X+1) transformed to correct for unequal variance and departures from normality, and means were separated using Tukey HSD Post Hoc Test at $p = 0.05$.

RESULTS

We detected MB from most of the samples, including control bark incubated with sterile agar, but there were large quantitative differences between treatments ($F = 6.99$, $p < 0.01$) (Figure 1A & 2). The highest levels of MB were detected in vials incubated with the two *Grosmannia* species, with *G. europhioides* emitting 35.5 × more MB than the control ($p < 0.01$) and *G. penicillata* emitting 10.1 × more MB ($p < 0.01$) 7 days after incubation. Incubation with *E. polonica* or *O. piceae* yielded low amounts of MB that did not differ significantly from the sterile agar control ($p = 0.92$ for *E. polonica* and 0.06 for *O. piceae*).

In addition to the quantitative differences there were also large temporal differences in MB release between treatments: MB was detected a few hours after incubation from most samples with *G.europhioides* and *G. penicillata*, but not until three days after incubation with *E.*

polonica, *O. picea* or sterile agar. The highest levels of MB were detected 7 days after incubation (Figure 2).

To determine if MB was produced *de novo* by the fungus or if it was simply a byproduct of fungal degradation of spruce tissues, we incubated *G.europhioides* on malt agar. MB was detected in all three replicates with *G.europhioides* growing on malt agar, but not from the sterile agar control, suggesting that MB was *de novo* produced by the fungus. To confirm the biosynthetic origin of MB, we incubated *G. europhioides* in vials with malt agar containing 0.5% ^{13}C labeled glucose. Labeled glucose was clearly incoporated into MB sampled in the headspace above the fungus seven days after incubation (Figure 1B), showing that *G. europhioides* can use glucose as a carbon source to produce MB.

DISCUSSION

In this study we have demonstrated that the bark beetle symbiont *G.europhioides* and probably *G. penicillata* can produce MB *de novo*, using glucose as a carbon source. To our best knowledge, this is the first demonstration of *de novo* pheromone synthesis by a bark beetle-associated fungus. Fungi generally dissimilate organic compounds such as glucose through one or more glycolysis pathways and then go on to produce various alcohols, ketones and benzenoids through fermentation or heterotrophic pathways (Davis et. al 2013). The biosynthesis pathway for MB production in fungi is completely unknown, as MB are rarely reported from *Grosmannia* or any other microbial source.

Bark beetle-associated microbes have previously been demonstrated to be involved in the production of behaviour-regulating chemicals. Hulcr et al. (2011) observed that ambrosia beetles are attracted to volatiles from their fungal symbionts under field conditions, but the chemical substances involved were not identified. Other examples with a chemical perspective include the *in vitro* conversion of the tree defense compound α-pinene to *cis*-verbenol (an aggregation pheromone component of several bark beetles) by the bacterium *Bacillus cereus* isolated from the gut of California fivespined ips *Ips paraconfusus* (Brand et. al 1975), and the interconversion of verbenol and verbenone (an anti-aggregation pheromone) by yeasts associated with the spruce bark beetle and the mountain pine beetle *Dentroctonus ponderosae*, as well as a fungus associated with the southern pine beetle *D. frontalis* (Brand et. al 1976; Hunt and Borden 1990; Leufven et. al 1984). Ingestion of an antibiotic inhibited production of ipsenol and ipsdienol in male *I. paraconfusus*, suggesting that gut microbes may be directly or indirectly involved in the production of these pheromone components (Byers and Wood 1981). However, none of these previous studies have presented conclusive evidence from e.g. labelling experiments showing that beetle-associated microbes produce bark beetle pheromones *de novo*.

MB is the most abundant aggregation pheromone component of the spruce bark beetle (Lanne et. al 1989). It is also an aggregation pheromone component of several other *Ips* bark beetles and an alarm pheromone in the European hornet *Vespa crabro* (Zhang et. al 2012). MB is a five-carbon hemiterpenoid alcohol that was originally believed to be derived by the spruce bark beetle from precursors in the host bark, but has since been demonstrated to be produced *de novo* by the beetles (Lanne et. al 1989). Male beetles incorporate ^{14}C into MB in the gut following injection of ^{14}C labelled glucose, acetate or mevalonate into the subcuticle. The fact that mevalonate injection increase radioactivity of MB, suggests that MB is *de novo* produced by the beetles in the gut via the mevalonate pathway. Still, it cannot be ruled out that microorganisms in the beetle gut or elsewhere are responsible for, or contribute to, MB production.

Not only insects and microbes, but also plants seem able to produce MB. MB emission is reported from the needles of several North American pine species (Harley et. al 1998) and from the bark of several birch and aspen species (Zhang et. al 2012). In digger pine *Pinus sabiniana* a bifunctional MB synthase has been identified that produces MB and isoprene in a ~90:1 ratio via the dimethylallyl diphosphate (DMADP) pathway (Gray et. al 2011). We also detected MB in small quantities in the headspace above our Norway spruce bark controls a few days after the bark plugs were taken from the log. However, since spruce bark may carry microbes capable of producing MB it is premature to conclude that Norway spruce tissues were the source of the MB we detected.

In conclusion, our finding that bark beetle-associated fungi produce substantial amounts of MB *de novo* suggests that these fungi may play a role in the aggregation of spruce bark beetles. It also provides new insights on the interaction and coevolution between insects and microbial symbionts. However, since fungal establishment and pheromone production may be slow relative to bark beetle mass-attacks, future studies are needed to determine how much the symbionts' chemical signaling contributes to bark beetle aggregation behavior in nature.

ACKNOWLEDGMENTS

This study was supported by grants from FORMAS (The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, Grant no: 229-2011-890) to Tao Zhao, and the Research Council of Norway (Grant no: 221479/F20) to Paal Krokene.

References

Hulcr J, Mann R, Stelinski LL. 2011. The scent of a partner: ambrosia beetles are attracted to volatiles from their fungal symbionts. Journal of Chemical Ecology 37(12):1374-1377.

Jankowiak, R. 2005. Fungi associated with *Ips typographus* on *Picea abies* in southern Poland and their succession into the phloem and sapwood of beetle-infested trees and logs. Forest Pathology 35(1): 37-55.

Kirisits, T. 2004. Fungal associates of European bark beetles with special emphasis on the Ophiostomatoid fungi. In F. Lieutier & E. Al. (Eds.), Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis (pp. 181–235). Kluwer Academic Publishers.

Krokene, P., & Solheim, H. 1996. Fungal associates of five bark beetle species colonizing Norway spruce. Canadian Journal of Forest Research 26:2115–2122.

Blomquist GJ, Figueroa-Teran R, Aw M, Song MM, Gorzalski A, Abbott NL, Chang E, Tittiger C. 2010. Pheromone production in bark beetles. Insect Biochemistry and Molecular Biology 40(10):699-712.

Brand J, Bracke J, Britton L, Markovetz A, Barras S. 1976. Bark beetle pheromones: Production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. Journal of Chemical Ecology 2(2):195-199.

Brand J, Bracke J, Markovetz A, Wood D, Browne L. 1975. Production of verbenol pheromone by a bacterium isolated from bark beetles. Nature 254:136-137.

Byers J, Wood D. 1981. Antibiotic-induced inhibition of pheromone synthesis in a bark beetle. Science 213(4509):763-764.

Connor MR, Liao JC. 2008. Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. Applied and Environmental Microbiology 74(18):5769-5775.

Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK. 2013. Microbial volatile emissions as insect semiochemicals. Journal of Chemical Ecology 39(7):840-859.

Franceschi VR, Krokene P, Christiansen E, Krekling T. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytologist 167(2):353-375.

Gray DW, Breneman SR, Topper LA, Sharkey TD. 2011. Biochemical characterization and homology modeling of methylbutenol synthase and implications for understanding hemiterpene synthase evolution in plants. Journal of Biological Chemistry 286(23):20582-590.

Harley P, Fridd-Stroud V, Greenberg J, Guenther A, Vasconcellos P. 1998. Emission of 2-methyl-3-buten-2-ol by pines: A potentially large natural source of reactive carbon to the atmosphere. Journal of Geophysical Research: Atmospheres (1984–2012) 103(D19):25479-486.

Hunt D, Borden J. 1990. Conversion of verbenols to verbenone by yeasts isolated from *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Journal of Chemical Ecology 16(4):1385-1397.

Krokene P. 2015. Conifer defense and resistance to bark beetles. In: Fernando E. Vega RWH, editor. Bark Beetles: Biology and Ecology of Native and Invasive Species.Science Direct. p 177-207.

Lanne BS, Ivarsson P, Johnsson P, Bergstrom G, Wassgren AB. 1989. Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera, Scolytidae) Insect Biochemistry 19(2):163-167.

Leufven A, Bergstrom G, Falsen E. 1984. Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus*. Journal of Chemical Ecology 10(9):1349-1361.

Raffa KF, Aukema BH, Bentz BJ, Carroll AL, Hicke JA, Turner MG, Romme WH. 2008. Cross-scale drivers of natural disturbances prone to anthropogenic amplification: The dynamics of bark beetle eruptions. Bioscience 58(6):501-517.

Renwick J, Hughes P, Krull I. 1976. Selective production of *cis*-and *trans*-verbenol from (-)- and (+)-alpha by a bark beetle. Science 191(4223):199-201.

Zhang Q-H, Schlyter F, Birgersson G. 2012. 2-methyl-3-buten-2-ol: a pheromone component of conifer bark beetles found in the bark of nonhost deciduous trees. Psyche: A Journal of Entomology 2012.

Figure legends

Figure 1. (A). Representative chromatograms using a HP-5 column showing 2-methyl-3-buten-2-ol (MB) released from bark with sterile agar and agar colonized by *Grosmannia penicillata* and *G. europhioides.* (B). Representative mass spectra showing incoporation of ^{13}C into 2-methyl-3-buten-ol (MB) produced by *Grosmannia europhioides* growing on malt agar with 0.5% unlabeled glucose or ^{13}C labeled glucose.

Figure 2. 2-Methyl-3-buten-2-ol (MB) emission from Norway spruce bark incubated with sterile malt agar or agar colonized by each of four fungal associates of the spruce bark beetle *Ips typographus* 1-7 days after incubation. Data are expressed as means ± 1 SE (n = 6). Stars indicate significant differences from the control by repeated measures one-way ANOVA.

Figure 1

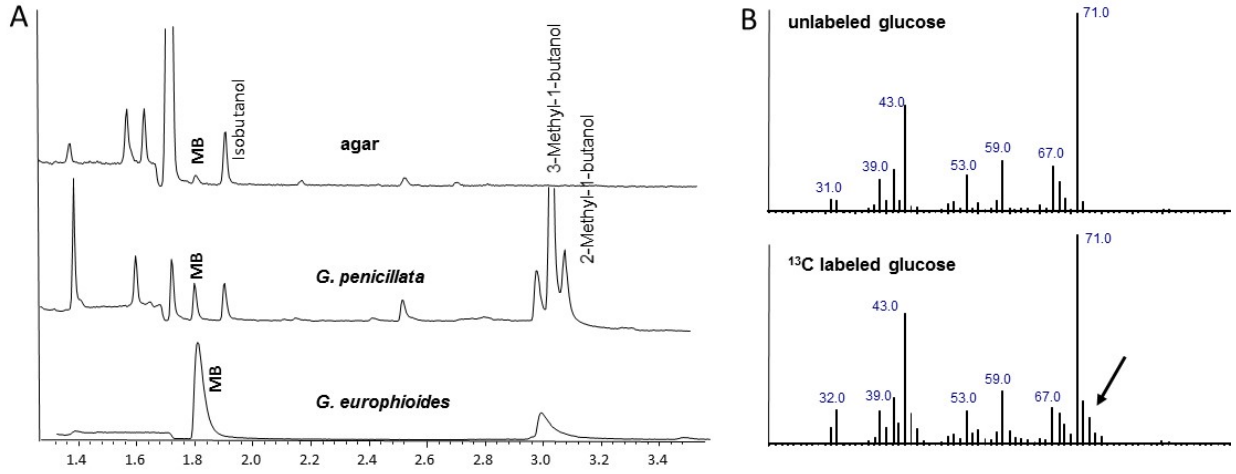

Figure 2